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Potential endocrine disrupting properties of toys for babies and infants

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Keywords:	endocrine activity, children, toys, migration, risk assessment
Abstract:	Plastic toys mouthed by children may be a source of exposure to endocrine active substances. The purpose of this study was to measure hormonal activity of substances leaching from toys and to identify potential endocrine disruptors causing that activity. For this purpose, migration experiments of toys were conducted in saliva simulants. The CALUX® assays were used to detect (anti-) estrogenic and (anti-) androgenic activity of 18 toys. Chemical trace analysis – namely, GC-MS and HPLC-MS- was used to identify which compounds may be responsible for endocrine activity in the sample migrates. Nine out of 18 tested toys showed significant estrogenic activity. For two samples, the detected estrogenic activity could be well explained by detecting the known endocrine active substance bisphenol A (BPA). For all identified substances, including BPA, a risk assessment for human health was performed by comparing the exposure dose, calculated based on the determined substance concentration, to toxicological reference values. Using worst-case scenarios, the exposure to BPA by mouthing of the two estrogen active, BPA-containing toys could be above the temporary TDI that EFSA has calculated. This demonstrates that some toys could significantly contribute to the total exposure to BPA of babies and infants.
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Potential endocrine disrupting properties of toys for babies and infants

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Introduction

Children, in particular, those below the age of 36 months, are considered particularly vulnerable to chemical substance exposure, since the ratio between exposure and body weight is different between children and adults [1]. Furthermore, developing periods constitute windows of susceptibility to some compounds showing endocrine activity. This may explain the increased incidence of certain diseases such as neurodevelopmental disorders or effects on the reproductive tract [2].

Children are exposed in their daily life to multiple chemical compounds, via food, dust, personal care products and other consumer products [3,4]. Several studies based on observations on the mouthing behaviour of children from 0 to 36 months confirm that children put a great diversity of objects into their mouths, including toys [1]. Plastic toys account for the majority of toys purchased in France. However, only few studies have assessed the risks associated with chemical substances present in plastic toys and children's equipment intended for infants and children up to

three years [5]. Plastic toys are often made with complex mixtures of one or more polymers combined with multiple additives such as plasticizers, flame retardants, antioxidants …. As some of these constituents are not covalently bound to the polymers, plastics have been shown to release chemicals such as phthalates or UV filters that are known endocrine disrupters [4].

Toys are regulated in the European Union by a Directive which stipulates that toy manufacturers shall carry out an analysis of the chemical, physical, mechanical, electrical, flammability, hygiene and radioactivity hazards that the toy may present and assess the potential exposure to these hazards. In terms of chemical safety, the directive prohibits the use of carcinogenic, mutagenic and reprotoxic (CMR) substances in categories 1A, 1B or 2 in toys or structurally separate parts, except if the substance is inaccessible or present in concentrations below a certain threshold. Plastic materials have been suggested as a relevant source of human exposure to endocrine disrupting chemicals (EDCs) [6-8].

Today, even if a common definition of what is an endocrine disrupter has been adopted at the European level, there is no regulatory obligations for those placing toys on the market to address this concern for endocrine effects yet. On the contrary, in the recently published Medical Device Regulation 2017/745, it is stated that substances having endocrine-disrupting properties with scientific evidence of probably serious effects on humans, shall only be present in a medical device above 0.1% by weight (w/w) when justified.

Phthalates are a group of substances, which have been widely used in toys as plasticizers to increase their flexibility. Several phthalates have been classified as reprotoxicants 1B and therefore been banned in toys or other articles which may be put into the children mouth. For example, the use of di(2-ethylhexyl) phthalate (DEHP), Di-n-butylphthalate (DnBP), Di-iso-butylphthalate (DiBP) and Butylbenzylphthalate (BBP) is restricted to a maximum level of 0.1% in all toy plastic parts following entry 51 of Annex XVII to REACH [9]. In addition, the same maximum threshold has to be applied to Di-iso-nonyl-phthalate (DiNP), di-n-octyl phthalate (DnOP), di-iso-decyl phthalate (DiDP) when children can place toys in their mouth (entry 52 of Annex XVII to

REACH). Some phthalates have also been recently identified as endocrine disrupters under the REACH regulation [10].

Actually, the described low regulatory limit of 0.1 % is equivalent to a ban of phthalates in plastics, as a minimal level 10% phthalates is required to achieve the softening effect on PVC. As a result, efforts have been made by industry to substitute many of the reprotoxic phthalates with less potent substances, such as Bis-2-ethylhexyl terephtalate (DEHTP) or Acetyl tributyl citrate (ATBC), in toys. However, for many substitutes endocrine activity data is not available. Other compounds used in plastics such as Bisphenol A (BPA) have also been recently identified as endocrine disrupters under the REACH regulation [10].

In addition to chemicals with known endocrine disrupting potential, toys may contain substances that have never been evaluated for their endocrine activity. Especially for non-intentionally-added substances, such as degradation products or contaminants, little is known on their potential endocrine effects. To be able to detect not only known or suspected endocrine disruptors, but also currently unknown substances with an endocrine activity, biodetection systems can be used [11,12]. Berger et al. [6] have detected significant endocrine activity in two out of eleven tested plastic teethers using Yeast Estrogen and Yeast Androgen screen assays. The estrogenic and antiandrogenic activity of one of these teethers was linked to methyl- and propyl paraben. Using similar approaches, endocrine activity was reported for several plastic food contact materials ([4, 13-16], canine plastic toys [17] and water from plastic bottles [8].

Thus, the purpose of this study was to analyse selected toys for leaching of endocrine active substances potentially absorbed by toy-mouthing children. Several studies have already been carried out to assess the health risks associated with substance exposure originating from toys [18–21]. Most of these studies, however, assessed exposure scenarios based on the composition as well as migration or emission tests to identify hazardous chemicals that need to be avoided or reduce in the material composition.

Hence, in the present study, toys and children's equipment intended for children up to three years were screened for EDCs using effect-directed analysis [23]. The CALUX® assays (Chemically Activated Luciferase gene expression) were applied to detect substances (i.e. ligands) interacting with the human estrogen or androgen receptors and thereby modifying the subsequent transcriptional response [24, 15].

In a previous study, composition and migration tests were already conducted on these items, in order to search for plasticizers (phthalates and their substitutes[1]) [5]. Out of 31 toys and items of children's equipment tested, only the PVC toys contained plasticizers, with the exception of one elastomer toy containing ATBC. The plasticizers found included two prohibited phthalates (DEHP used as a principal plasticiser associated with DINP in a toy purchased at a street market, and low levels of DEHP attributed to its presence as an impurity of DEHTP in three toys), and some of their substitutes (ATBC, DEHTP, DINCH, DOIP, TXIB) were quantified. Subsequently, it was shown that all the substances found migrated in a saliva simulant.

The current work focuses on the analysis of toy migrates using bioassays and known or suspected hormone active substances were screened by GC-MS and HPLC-MS/MS. The potential hormonal activities of these toys have been assessed.

1. MATERIAL AND METHODS

1.1. Selection of toys

A literature review focusing on extensive reports and surveys of professionals from the toy sector was performed to identify the materials and compounds likely to be most commonly used in toys in France and Europe [5]. The categories of toys sold most widely in the EU are pre-school and infant

[1] Any substance that plays a technological role similar to that of phthalates in the materials.

toys, followed by dolls, outdoor toys and board games/puzzles (more than half of all sales in the EU).

Toys and children's equipment represent a wide diversity of types. Their materials (hard/soft plastic, wood, textiles, metals, *etc.*) and the substances they are composed of vary in a great extent. Without this being an exhaustive list, they include the following substances: phthalates and substitutes, flame retardants, short-chain chlorinated paraffins, bisphenol A, metals, *etc.* In France, the best-selling toys over all age groups are construction toys, dolls, board games and puzzles, infant toys and outdoor toys. Taking into account only toys made of plastic and the target population (children up to 36 months), this work focused on the following toy categories: infant toys, dolls and construction toys.

In a previous study, a set of toys has been analysed for its concentrations of several endocrine active substances: tests were carried out in France to characterize toys present on the French market for which leaching of hazardous compounds may expose young children. Tests of the composition of phthalates and their substitutes in a limited sample of toys and children's equipment (bibs, teething rings, pacifiers), followed by migration testing in a saliva simulant were conducted on ANSES's request [5]. These tests were only conducted on new toys. Based on the results of these tests, 18 toys were selected by Anses for further testing to assess endocrine activity of the migration fluid. Table 2 shows the samples that were selected for analysis and ordered online.

1.2. Characterization of materials

Toy samples were characterized following a 3-step approach. First, the toy itself, its packaging and the producers' web sites were examined for available product information (e.g. recycling codes). If not enough information could be obtained, a formal request asking for the used raw materials was directly sent to the toy manufacturer as a second step. Several toy producers agreed to share this information (see table 2). In a third step the remaining samples were analysed by Fourier-transform

infrared (FT-IR) spectroscopy analysis using a FT-IR-spectrometer type Perkin-Elmer, model Spectrum One to identify the materials. As a general approach the samples were measured on the surface as well as in the internal part. Recordings were made using a universal ATR unit (equipped with Diamond/ZnSe crystal) in a spectral range from 4000-600 cm^{-1}.

1.3. Migration experiments

To simulate the process of toy mouthing by children, the toy subparts were incubated with saliva simulant (0.82 mM $MgCl_2$ (Sigma), 1 mM $CaCl_2$ (VWR), 3.3 mM K_2HPO_4 (Sigma), 3.8 mM K_2CO_3 (Sigma), 5.6 mM NaCl (Sigma), 10 mM KCl (Sigma), pH of 6.8 – stored in the dark for 2 weeks). Migration experiments were conducted based on the JRC Scientific and Technical Report JRC EUR 23813 EN (2009) [25]. If possible, toys were migrated without any further cutting in a wide-neck 1000 ml glass bottle closed tightly with a polytetrafluoroethylene (PTFE) cap applying saliva simulant in a ratio of 100 ml per 10 cm^2 sample surface. Samples that did not fit into wide-neck 1000 ml glass bottles, were cut into pieces of 20 to 47 cm^2 adjusting the volume of saliva simulant accordingly to obtain a ratio of 100 ml per 10 cm^2. Some toys were split into different subsamples and analysed independently as they were composed of different materials. For instance, a doll was split into three subsamples: dress, hair and face. For comparison, saliva blanks, filled in glass bottles with PTFE coated caps, were migrated to check for possible contaminations of the saliva simulant. During migration, bottles were rotated on a head over heels rotator at 60 rpm at room temperature. After 30 min of rotation, the simulant was collected and replaced by fresh saliva again incubating with the sample for 30 min. Then, both migration batches were collected and pooled reaching a total volume of 400 ml. Simulants were stored at 4 to 8°C.

After migration, SPE with Oasis HLB columns (6 cc/200 mg) was carried out to concentrate the obtained migrates. The preparation procedure of the columns included conditioning with 15 ml of an ACN-MeOH 1:1 mixture and 5 ml MeOH, followed by equilibration with 5 ml ultrapure water. After the samples were applied to the column using vacuum (around 0.7 bar), each column was washed with 5 ml ultrapure water and eluted with 4.5 ml of an ACN-MeOH 1:1 mixture. 200 µl

DMSO was added to each vial as a “keeper”, before the samples were eluted and evaporated to a final volume of 250-300 µl at vacuum (between -0.2 and 0.7 bar) and stored at 4°C prior to analysis by the bioassays.

All sample treatment procedures have been previously validated for recovery and reproducibility using 10% ethanol migrates [15]. To demonstrate that the developed methods are suitable for the concentration of saliva solvent, recoveries and reproducibility were determined as previously described by Mertl et al., [15]. 1 liter of saliva solvent was spiked with 2,4-dihydroxybenzophenone, bisphenol A, benzophenone and benzylbutylphthalate at a concentration of 10 ppb and extracted by SPE and transferred to DMSO. Concentrations of the spiked substances were determined by HPLC-UV/VIS in the concentrated DMSO extracts.

1.4. Analysis of toys for hormonal activity with different *in vitro* tests

The DMSO extracts were analysed by two different CALUX® bioassays (Estrogen Receptor α-CALUX® and Androgen receptor-CALUX®). CALUX® (Chemical Activated Luciferase Expression) assays are human cell-based reporter gene assays for the detection of hormonal activity. In more detail, these assays measure estrogen/androgen and anti-estrogen/anti-androgen activity as previously described by Mertl et al., [15]. Both bioassays are based on a human U2-OS osteosarcoma cell line and show a highly sensitive and selective response to natural and synthetic estrogen- or androgen agonists. In the modified U2-OS cells, an activated steroid hormone receptor binds to the promoter region of a firefly luciferase reporter gene and activates firefly luciferase transcription. The firefly luciferase reporter gene leads to a light emission if luciferin and co-factors are added and can be very sensitively and selectively quantified. This light signal increases depending on the dose of estrogen or androgen-active substances (Van der Burg 2010).

Three independent migrates of each sample extract were analysed in triplicates in both CALUX®-assays. The concentrated DMSO extracts were added to a final concentration of 0.5% to the assay medium. The activity was quantified by comparison to a linear regression curve of a dilution series

of the natural estrogen 17β-estradiol or androgen 5α-dihydrotestosterone (DHT). In addition, all samples were further tested for cytotoxic effects or growth inhibiting effects by microscopy.

To test for antagonisms or inhibiting effects by the sample matrix, each sample was spiked with a non-saturating concentration of a suitable positive control (8 pmol/l of 17β-estradiol or 400 pmol/l of DHT) and analysed via the respective CALUX® assay. If more than 40% of the activity of the spiked hormone was suppressed by the tested sample, the samples have to be diluted prior to analysis for hormone agonists, resulting in increased detection limits. Samples, which show such a significant suppression of the hormone activity of the spiked hormone, were categorized as antagonistic, if cytotoxic effects cannot explain the reduction of the activity.

Reproducibility of the CALUX® assays and robustness towards polymer extracts was determined in a previous study [15].

1.5. Chemical trace analysis

The toys were further tested to identify which compounds may be responsible for reported hormonal activities. Chemical trace analysis (GC-MS and HPLC-MS/MS) was performed on 34 toys or toy parts. Sample migrates were analysed for 41 known or suspected endocrine active substances (e.g. Bisphenol A, phthalates and parabens) as well as common alternatives to endocrine active plasticizers (e.g. TXIB, ATBC).

Gas chromatography analysis was carried out as previously described in Mertl et al. [15] extending the substance set for additional target substances (see Table 1). In short, a Stir Bar Sorptive Extraction (SBSE) using PDMS (polydimethylsiloxane) GERSTEL twisters was performed, followed by thermal desorption and GC-MS analysis. 5 ml of each saliva migrate were extracted for 1 hour on a magnetic stirring plate at 2000 rpm. The removed twister was dried and placed in a thermal desorption tube. Identification and quantification of 28 target compounds as already described in Mertl et al. [15] adding 10 additional substances was done applying selected ion monitoring (SIM) mode. For each reference substance a calibration curve was determined for concentrations

between 1 µg/l and 50 µg/l and LODs and LOQs were evaluated according to DIN 32 645. For the identification of unknown compounds SCAN mode using the NIST02 library (US National Institute of Standards and Technology) followed by comparison to known mass spectra was conducted. Two independent migrates were separately analysed in duplicates. Conditioning of the twisters at 300 °C for ten minutes was done to avoid contaminations from previously tested samples.

Besides GC-MS analysis, concentrations of Bisphenol A, F and S were measured with high-pressure liquid chromatography combined with tandem mass spectrometry (Dionex U3000 HPLC, AB-Sciex Qtrap 5500), using ESI (electrospray ionization) for ionization and injection on the ACE 3 C18-AR 150 x 3 mm (V13-7639) column.

Measurements with a negative multi-reaction-monitoring (MRM)-mode show two different stable transitions (parent ion – fragment ion) for each analyte, which were used for qualification and quantification, respectively. For quantification, peak areas were integrated using Analyst 1.6 and an external calibration. The limits of detection and quantification were determined statistically according to DIN 32465.

Table 1: Limits of detection and quantification in the GC-MS and HPLC-MS/MS screening for the target compounds (the LODs and LOQs refer to the migrate solution)

Substance	CAS - Number	Limit of detection [µg/l]	Limit of quantification [µg/l]	Method
1,4-Dichlorobenzene	106-46-7	0.5	1.8	GC-MS
4-chloro-3-methyl-phenol	59-50-7	1.0	3.6	
Methyl p-hydroxybenzoate (Methylparaben)	99-76-3	0.7	2.6	
Butylated hydroxyanisole (BHA)	25013-16-5	0.9	3.2	
2,4-di-tert-Butylphenol	96-76-4	1.0	3.7	
2-Phenylphenol	90-43-7	1.2	4.4	
Ethyl-4-hydroxy-benzoate (Ethylparaben)	120-47-8	1.4	5.0	
Diethyl phthalate	84-66-2	1.1	3.8	
n-Propyl-p-hydroxybenzoate (Propylparaben)	94-13-3	0.8	2.9	
Benzophenone	119-61-9	1.1	3.7	
1,3-Diphenylpropane	1081-75-0	1.1	3.9	
4-Phenylphenol	92-69-3	0.8	2.7	
4-Methylbenzophenone	134-84-9	0.8	3.0	
trans-1,2-Diphenylcyclobutan	20071-09-4	1.1	3.9	
p-Cumyl phenol	599-64-4	0.5	1.7	
4-Nonylphenol (NP)	104-40-5	1.1	4.0	

Dibutyl phthalate (DBP)	84-74-2	0.8	2.9	
Oxybenzone	131-57-7	0.5	1.7	
Triclosan	3380-34-5	0.7	2.4	
2,4-Dihydroxybenzophenone	131-56-6	0.5	1.6	
Tributyl citrate (TBC)	77-94-1	1.2	4.4	
2,20-Dihydroxy-4-methoxybenzophenone	131-53-3	0.7	2.3	
Di-2-ethylhexylfumarat	141-02-6	1.5	5.0	
Acetyl tributyl citrate (ATBC)	77-90-7	0.9	3.1	
Di-n-hexylphthalate (DnHP)	84-75-3	0.5	1.9	
Butyl benzyl phthalate (BBP)	85-68-7	0.5	1.8	
Oleamide	301-02-0	1.0	3.3	
Bis(2-ethylhexyl) adipate (DEHA)	103-23-1	0.6	2.1	
2,2´-Methylene bis(4-methyl-6-tert-butylphenol)	119-47-1	0.6	2.1	
2,2´-Methylenebis(4-ethyl-6-tert-butylphenol)	88-24-4	0.6	2.2	
Dicyclohexyl phthalate	84-61-7	0.6	2.0	
2,4-Dicumylphenol	2772-45-4	0.5	1.7	
Bis (2-ethylhexyl) phtalate (DEHP)	117-81-7	0.3	1.1	
Bis-2-ethylhexyl isophthalate (DOIP)	137-89-3	0.8	2.7	
Diphenyl-p-phenylenediamine	74-31-7	0.5	1.8	
DEHTP (Bis-2-ethylhexyl terephtalate)	6422-86-2	0.8	2.7	
4,4´-Thiobis(6-terc-butyl-3-methyl-phenol)	96-69-5	0.6	1.9	
(bis-2-propylheptyl phthalate) DPHP	53306-54-0	0.6	2.1	
Bisphenol A	80-05-7	0.4	1.6	HPLC-MS/MS
Bisphenol F	620-92-8	2.2	8.0	
Bisphenol S	80-09-1	1.4	5.2	

Figure 1 summarizes how the endocrine potential of these toys has been investigated.

Figure 1 : Scheme of sample analysis

1.6. Analysis of detected pure substances for endocrine activity

Substances detected by chemical target analysis (TBC, benzophenone and diethyl phthalate) in at least one sample were used to prepare a dilution series (100 mM, 30 mM, 10 mM, 3 mM, 1 mM, 0.3 mM and 0.1 mM) in DMSO, subsequently tested for endocrine activity in the ER CALUX® assay. Testing procedures followed instructions given in 1.4.

1.7. Assessment of the health risks associated with the mouthing of plastic toys containing phthalate substitutes

Health risk assessment was performed for critical substances detected in the selected toys - the two phthalate substitutes acetyl-tributyl-citrate (ATBC, n° CAS 77-90-7), and diethylhexyl-terephthalate (DEHTP, n° CAS 6422-86-2) as well as for other potential active substances such as tributyl citrate (TBC, n° CAS 77-94-1), Bisphenol A (CAS 80-05-7) and Benzophenone (CAS 119-61-9).

Exposure scenarios adapted from RIVM [19] were applied to estimate oral exposure from mouthing behavior of toys in three subpopulations of children (0 to 12 months, 13 to 24 months and 25 to 36 months) to consider specificities in exposure parameters.

The following equation was used to calculate the daily exposure (DE) to the substances:

Equation 1:

$$DE = \frac{F \times S \times D}{BW} \quad (1)$$

With:

- BW : body weight [kg]
- F : migration flux [µg/min/cm²]
- S : surface in contact with the mouth [cm²]
- D : contact duration [min/j]

Risks were estimated by comparing the daily exposure (DE) to toxicological reference values (TRV) when available.

2. RESULTS AND DISCUSSION

2.1. Selected toys and characterization of materials

In a 3-step approach (research, contact to the manufacturer and FT-IR analysis) the material of the tested toys was characterized. Table 2 gives an overview on all selected toys and sampling areas and lists the determined material of all tested sample parts.

Table 2: samples selected for analysis and material characterization

Sample code	Game	Age class	Sampling area	Material
T01	Bath book	From 4 months	Plastic part of page	EVA [(b)]
T02	Plastic toy	12 months - 6 years	Plastic	PVC [(a)]
T03a	Book early age	Since birth	Fabric	Fabric
T03b			Battery lid	ABS [(a)]
T04a	First age toy	From 6 months	Support	Styrene acrylate ; core: PP/PE [(a)]
T04b			Orange ring	EVA [(a)]
T04c			Yellow ring	EVA [(a)]
T05a	Bath game	unspecified	Star	PVC [(a)]
T05b			Hippo	PVC [(a)]
T06a	Teething key ring	From 3 months	Blue key	PE [(a)]
T06b			Ring	PP-PE co-polymer[(a)]
T07a	Doll	From 4 years	Dress	Fabric
T07b			Hairs	Unknown
T07c			Face	PVC [(a)]
T08a	Doll	From 3 years	Arm	PVC [(a)]
T08b			Battery lid	ABS [(a)]
T08c			Fabric	Fabric
T09a	Doll	From18 months	Arm	PVC[(a)]
T09b			Fabric	Fabric
T10a	Building game	From18 months	Red brick	ABS[(b)]
T10b			Yellow brick	ABS[(b)]
T11a	Building game	12 months – 5 years	Yellow brick	ABS[(b)] with sticker
T11b			Green brick	ABS[(b)]
T12a	Building game	From18 months	Cone	Wood
T12b			Triangle	Wood

T13a	Figure	From 18 months	Tailgate	ABS/steel[(b)]
T13b			Wheel	ABS/steel[(b)]
T14a	Outdoor play equipment	From 18 months	Ball	Polyurethane coating – PVC [(a)]
T14b			Bucket	PP [(b)]
T14c			Shovel	PP [(b)]
T15	Ball	From 6 months	Soft shell	Polyurethane coating – PVC [(a)]
T16	Soft toy	unspecified	Fabric	Fabric
T17	Soft toy	Since birth	Fabric	Fabric
T18	Soft toy	Since birth	Fabric	Fabric

256

257 **2.2. Endocrine activity of the toys migrates**

258 To test the endocrine potential of selected toys, saliva migrates were prepared from each sample.
259 Prior to bioassay analysis migrates have been concentrated. A suitable solid phase extraction pre-
260 concentration method for 10% ethanol migrates was already validated for recovery and
261 reproducibility by Mertl et al. [15]. To verify the method applicability on saliva migrates, re-
262 validation experiments were performed. Saliva simulant was spiked with 4 representative reference
263 substances to determine substance losses during SPE concentration. Targeted HPLC-UV/VIS
264 analysis determined reasonable compound recoveries (benzophenone: 61 ± 5 %,
265 benzylbutylphthalate: 92 ± 4 %, bisphenol A: 83 ± 10 and2,4-dihydroxybenzophenone: 83 ± 3 %)

266

267 After simulating mouthing by a child, the saliva simulant was concentrated by solid phase
268 extraction, transferred to DMSO and analysed using CALUX® bioassays. These biological assays
269 can detect substances that can activate or inhibit the human estrogen or androgen receptor,
270 including currently unknown hormone active substances.

271

272 The results of the bioassay screening are listed in Table 3. Nine out of 18 tested toys showed a
273 significant estrogenic activity in at least one of the tested parts. The detected estrogenic activities
274 ranged from 5.3 to 83 pg estradiol equivalents (EEQ)/cm² sample surface.

No androgenic activity could be detected in any of the tested samples.

To evaluate if the sample matrix of the toy migrates has an inhibiting effect on the biodetection and to check for hormone antagonists, all samples were spiked with the natural hormones in non-saturating concentrations. Test responses of spiked sample migrates were compared to spiked solvent blanks. All tested samples showed the expected response to the natural hormones - no significant inhibition of the estrogenic or androgenic activity was observed. This demonstrates, that the test worked properly in the presence of the sample matrix and shows no indication for estrogen or androgen antagonists in the samples.

Table 3: ***Estrogenic and androgenic activity of*** **the selected toys (mean ± standard deviation of the analysis of three independent samples)**

	ER-CALUX®		AR-CALUX®	
Sample code	**Estrogenic activity [pg EEQ/cm²]**	**Anti-estrogen activity**	**Androgen activity (pg AEQ/cm²)**	**Anti-androgen activity**
T01	< 4	No	< 120	No
T02	83 ± 7	No	< 120	No
T03a	< 4	No	< 120	No
T03b	< 4	No	< 120	No
T04a	< 4	No	< 120	No
T04b	< 4	No	< 120	No
T04c	< 4	No	< 120	No
T05a	54 ± 27	No	< 120	No
T05b	67 ± 22	No	< 120	No
T06a	< 4	No	< 120	No
T06b	< 4	No	< 120	No
T07a	< 4	No	< 120	No
T07b	< 4	No	< 120	No
T07c	< 4	No	< 120	No
T08a	< 4	No	< 120	No
T08b	< 4	No	< 120	No
T08c	22 ± 10	No	< 120	No
T09a	< 4	No	< 120	No
T09b	14 ± 7	No	< 120	No
T10a	< 4	No	< 120	No
T10b	< 4	No	< 120	No
T11a	< 4	No	< 120	No
T11b	< 4	No	< 120	No
T12a	< 4	No	< 120	No
T12b	< 4	No	< 120	No
T13a	< 4	No	< 120	No
T13b	< 4	No	< 120	No
T14a	2.6 ± 4.5 (1 of 3 extracts positive)	No	< 120	No
T14b	< 4	No	< 120	No
T14c	< 4	No	< 120	No
T15	< 4	No	< 120	No
T16	6.6 ± 2.2	No	< 120	No
T17	5.3 ± 1.8	No	< 120	No
T18	19 ± 17	No	< 120	No

The PVC sample T02 showed the highest estrogenic activity of all tested samples (83 ± 7 pg EEQ/cm²). An activity of 83 pg EEQ/cm² means that substances migrating from one square centimetre toy surface have the same estrogenic activity in the bioassay, as 83 pg of the natural female sex hormone 17β-estradiol. If plastic components or other industrial chemicals were responsible for the determined estrogen activity, a much higher concentration would be needed to explain the reported estrogenic activity, as estrogen mimicking substances are generally not fitting as well into the receptor-binding site as the hormone itself [26].

Based on the assumptions by RIVM [19] (surface of 10 cm² for 3 hours in contact with saliva) 2.5 ng estradiol equivalents were calculated as a maximum daily uptake for the sample with the highest estrogen activity (calculated from 0.083 ng EEQ/cm² detected in a 60 minute extraction to 2.5 ng EEQ in 10 cm² in 180 minute extraction time).

In comparison to estrogen activities taken up by food, the detected estrogen activities were low. Many food products naturally contain estrogens, for instance 23 ng/L of the natural female sex hormone 17β estradiol were analytically determined in cow milk by Courant et al. [27]. By orders of magnitude higher estrogen activities than in cow milk, can be found in soy bean rich food, that contains phytoestrogens. The maximum total daily uptake of estrogen activity by food is estimated to be up to 10,000 ng estradiol equivalents per day for babies who are fed by milk on soy bean basis [28]. The activities detected in the migrates of the saliva solvent were also significantly lower than estrogen activities previously reported for mineral water [7, 29, 30], where no estrogen activity would be expected.

That said, comparisons between *in vitro* estrogen activities in food and toys should be interpreted with caution, because the potential health effects depend highly on the substance causing the activity. Many aspects of endocrine action in the human body, like metabolization and half-life are

not taken into account in bioassays. Very importantly, some man-made estrogens have much higher half-lives in the human body. A prominent example is the contraceptive 17α ethinyl estradiol. It has approximatively the same *in vitro* estrogen activity as the natural sex hormone 17β-estradiol [31], but has, by orders of magnitude, higher activity in the human body, if taken up orally [32]. This is also reflected in the acceptable daily intake values calculated for these substances. For the natural estrogen 17β-estradiol the World Health Organisation calculated an acceptable daily intake (ADI) of 3 µg per day for an adult person. Referring to a child's body weight of 8 kg, 2.5 ng estradiol equivalents, which was calculated as the maximum uptake by the worst-case toy, would still be well below the ADI for the natural hormone 17β estradiol. For 17α ethinyl estradiol, however, an approximately 400-fold lower ADI of 7 ng for an adult person (60 kg) was calculated by Caldwell, Mastrocco et al. [33] based on working place thresholds. Therefore for the synthetic estrogen 17αethinyl estradiol the same activity would already be clearly above the calculated ADI for a child of 8 kg bodyweight.

Although the activity detected in the sample migrates was most probably neither caused by natural estrogens nor by 17α ethinyl estradiol these examples were described to illustrate that the nature of the responsible substance is influencing the hazard potential of an endocrine active sample. Consequently, no risk assessment is possible based on *in vitro* estrogen activities alone. Therefore, a chemical analysis was conducted in parallel to the *in vitro* assays.

2.3. Chemical trace analysis

To further investigate the substances causing the reported estrogenic activities in toy migrates, migrates were analysed by chemical trace analysis.

GC-MS and HPLC-MS/MS Target Screening

Table 4 lists the result from screenings for 41 target compounds by GC-MS and HPLC-MS/MS. Among them, six substances could be detected in at least one of the tested samples - that are: diethyl phthalate, benzophenone, TBC, ATBC, BPA and DEHTP. The remaining 33 substances could not be detected in any of the tested samples.

Two substances, of the six detectable ones, have known endocrine properties, namely, bisphenol A (BPA) and benzophenone [34,35].

BPA is a monomer used in the production of polycarbonate and epoxy resins and is an integral part of thermo ink paper. These materials are therefore considered as the main sources for exposure to BPA.

The two parts of toy “T05”, in which BPA could be detected, consist of neither of these materials, but of flexible PVC. BPA has previously been a common additive in PVC as a production aid to stabilize vinyl chloride monomer, but the use of BPA as a stabilizer for vinyl chloride monomer was stopped in Europe since 2001. BPA has also been restricted in food contact plastic in Europe. Nevertheless, BPA still seems to be used in the PVC production for toys [37] outside the scope of the European plastics regulation (EU) No 10/2011.The detected BPA concentrations in the samples T05a and T05b were well reproducible for three independently prepared migrations. With approximately 1 mg BPA migration per cm^2 sample surface, the migration is by orders of magnitude higher than migration from polycarbonate or epoxy materials to water or beverages [38]; even though the migration time and temperature were significantly lower in this study.

Besides “T05”, BPA could be detected in one out of three independently prepared migrates of sample T07b. However, the detected concentration was too low to be quantified.

Benzophenone was identified in five different tested sample parts (T02, T08a, T09a, T09b and T11a). Four of these are built from flexible PVC, one sample (T11a) is made of Acrylonitrile butadiene styrene (ABS) with a sticker of printed paper on it. In the latter one printing inks from the paper sticker seem to be the most plausible source for the detected benzophenone, as benzophenone is a common photo initiator in UV printing colors (IARC 2013). In PVC benzophenone is often us as a UV-stabilizer.

The 41 target compounds in the analysis include seven phthalates, previously widely used in plastic toys. Except for toy sample “T01”, where trace amounts of diethylphthalate were detected, none of the 18 tested toys leached significant amounts of these phthalates. In toys made of flexible PVC, the common phthalate alternatives TBC, ATBC, DEHTP and DINCH were detected. When simulating mouthing, these plasticizers leached into saliva solvent in concentrations up to 76 µg per cm^2 toy surface. While many phthalates are suspected to have endocrine disrupting potential, the detected alternatives are currently not considered as potential endocrine disruptors (Anses, 2016).

GC-MS Non-Target Screening

In addition to the target screening analysis for 41 substances, further substances could be identified in a GC-MS non-target screening by comparison of the mass spectra to the NIST 14 database (see Table 4).

The plasticizers DINCH and TXIB that are commonly used as phthalate alternatives could be identified in several PVC samples. Furthermore, *1-Propene-1,2,3-tricarboxylic acid, tributyl ester*

was identified in 6 samples. It is a less commonly used plasticizer and has been previously proposed as a safe alternative to phthalates [2].

Propanoic acid, 2-methyl-, 2-ethyl-3-hydroxyhexyl ester and *Propanoic acid, 2-methyl, 3-hydroxy-2,4,4-trimethylpentyl ester* were identified at the same retention time in different samples by comparison to NIST database. However, these identifications are assumed to be wrong assignments, as it seems more likely, that the detected peaks represent the TXIB hydrolysis product *(1-hydroxy-2,4,4-trimethylpentan-3-yl) 2-methylpropanoate.*

13-Docosenamide, detected in sample “T13b” and more commonly known as erucamide, is one of the most common slip agents in polyolefin and styrene polymerization. *13-docosenamide* is not endocrine active in the *in vitro* tests used in this study.

Oxiranecarboxylic acid, 3-methyl-3-phenyl-, ethyl ester,(cis- and trans-isomers) was identified in T07c. This identification was not confirmed by comparison to a reference substance, but it seems plausible, as the identified substance is used as compound in strawberry fragrances (Perflavory Information System 2015), and the tested sample part had a strawberry odor.

As pure substances were only available for DINCH, TXIB and 13-Docosenamide, none of the other identifications could be confirmed by comparison to reference standards.

406

407 **Table 4: Substances detected in HPLC-MS/MS and GC-MS screenings for 41 target**
408 **compounds (mean value +/- standard deviation of 3 independent sample migrates in µg/cm²**
409 **surface) and GC-MS non-target analysis**

410

Sample	Detected substance	CAS	concentration [µg/cm²]
T01	Diethyl phthalate	84-66-2	0.092 ± 0.011
	TXIB(2)	6846-50-0	no quantification
T02	Benzophenone	119-61-9	0.33 ±0.02
	DINCH(1)	166412-78-8	no quantification
	TXIB(1)	6846-50-0	no quantification
T03a	No substances identified	-	-
T03b	No substances identified	-	-
T04a	No substances identified	-	-
T04b	No substances identified	-	-
T04c	No substances identified	-	-
T05a	TBC	77-94-1	3.7 ± 0.4
	ATBC	77-90-7	76 ± 23
	BPA	80-05-7	1.3 ± 0.1
	1-Propene-1,2,3-tricarboxylic acid, tributyl ester(2)	7568-58-3	no quantification
	DINCH(1)	166412-78-8	no quantification
T05b	TBC	77-94-1	3.0 ± 0.2
	ATBC	77-90-7	54 ± 12
	BPA	80-05-7	1.1 ± 0.0
	DINCH(1)	166412-78-8	no quantification
T06a	No substances identified	-	-
T06b	No substances identified	-	-
T07a	1-Propene-1,2,3-tricarboxylic acid, tributyl ester (1)	7568-58-3	no quantification
T07b	BPA	80-05-7	1 out of 3 migrates: < LOQ, > LOD (2)
T07c	ATBC	77-90-7	0.083 ± 0.029
	Oxiranecarboxylic acid, 3-methyl-3-phenyl-, ethyl ester, (cis- and trans-) (2)	19464-92-7& 19464-95-0	no quantification
	1-Propene-1,2,3-tricarboxylic acid, tributyl ester(2)	7568-58-3	no quantification
	TXIB(1)	6846-50-0	no quantification
T08a	Benzophenone	119-61-9	12 ± 2
	TBC	77-94-1	0.26 ± 0.05
	ATBC	77-90-7	18 ± 5

	1-Propene-1,2,3-tricarboxylic acid, tributyl ester[2]	7568-58-3	no quantification
	TXIB[1]	6846-50-0	no quantification
T08b	No substances identified	-	-
T08c	1-Propene-1,2,3-tricarboxylic acid, tributyl ester[1]	7568-58-3	no quantification
	Propanoic acid, 2-methyl, 3-hydroxy-2,4,4-trimethylpentyl ester [2]	74367-34-3	no quantification
T09a	Benzophenone	119-61-9	0.57 ± 0.16
	TBC	77-94-1	2.2 ± 0.4
	ATBC	77-90-7	6.0 ± 2.3
	TXIB[1]	6846-50-0	no quantification
	Propanoic acid, 2-methyl, 3-hydroxy-2,4,4-trimethylpentyl ester [2]	74367-34-3	no quantification
	1-Propene-1,2,3-tricarboxylic acid, tributyl ester [2]	7568-58-3	no quantification
T9b	Benzophenone	119-61-9	0.38 ±0.14
	TXIB [1]	6846-50-0	no quantification
T10a	No substances identified	-	-
T10b	No substances identified	-	-
T11a	Benzophenone	119-61-9	0.36 ±0.21
T11b	No substances identified	-	-
T12a	Propanoic acid, 2-methyl-, 2-ethyl-3-hydroxyhexyl ester [2]	74367-31-0	no quantification
T12b	Propanoic acid, 2-methyl-, 2-ethyl-3-hydroxyhexyl ester [2]	74367-31-0	no quantification
T13a	No substances identified	-	-
T13b	13-Docosenamide, (Z)-[1]	112-84-5	no quantification
T14a	DEHTP	6422-86-2	12 ± 9
T14b	No substances identified	-	-
T14c	No substances identified	-	-
T15	DEHTP	6422-86-2	1.0 ± 0.1
	BPA	80-05-7	< LOQ
	TXIB[1]	6846-50-0	no quantification
T16	No substances identified	-	-
T17	No substances identified	-	-
T18	No substances identified	-	-

(1) Substance, identified in GC-MS non-target-screening, identification verified by comparison to an external reference standard

(2) Substance, identified in GC-MS non-target-screening, no verification of the identification

2.4. Correlation between bioassays results and chemical trace analysis

In order to identify the source of the hormonal activity in positively tested samples in the ER CALUX®, results from chemical analysis and bioassays were compared. For this purpose, all detected substances were tested as pure chemicals in the estrogen receptor CALUX®, if they were available. The response curves for the positively tested substances in the bioassay are shown in figure 2. From that the half-maximal effective concentration (EC50) was calculated for all compounds showing estrogenic effects in the ER-CALUX® (see table 5).

The determined estrogen activities of BPA, benzophenone and DEP were to be expected based on previously published results [2, 34, 35]. The phthalate alternatives TXIB, ATBC, DINCH and DEHTP were tested negative in the ER-CALUX®. Unexpectedly, the plasticizer TBC (Merck) showed a low, but significant estrogen activity at high concentrations, although the structure of the molecule would not suggest binding affinity to the estrogen receptor. It is possible, that this low estrogen activity, was caused by contaminants of this substance, rather than the substance itself, as the purity of the tested substance was below 97 %.

For substances, that could not be obtained as pure chemicals a literature survey was conducted. 1-Propene-1,2,3,-tricarboxilic acid, tributyl ester, propanoic acid and 2-methyl,3-hydroxy-2,4,4,trimethylpentyl ester are not registered yet under the REACH regulation. No data on the endocrine activity of these substances is available.

Figure 2: Analysis of detected pure substances in ER-CALUX®: Response of bisphenol A (BPA), diethylphthalate (DEP), tributyl citrate (TBC) and benzophenone in comparison to natural estrogen 17β-estradiol and a solvent blank. The two highest concentrations of TXIB led to a decrease in cell viability leading to decreased activities in the assay.

Table 5: EC_{50} values of detected pure substances in ER-CALUX®

Substance	CAS#	Estrogen active [yes/no]	EC_{50} [mol/l]
Diethyl phthalate (DEP)	84-66-2	yes	$2*10^{-4}$
2,2,4-Trimethyl-1,3-pentanediol diisobutyrate (TXIB)	6846-50-0	no	$> 5*10^{-4}$
13-Docosenamide (Z)	112-84-5	no	$> 5*10^{-4}$
Benzophenone	119-61-9	yes	$5*10^{-5}$
Diisononyl cyclohexane-1,2-dicarboxylate (DINCH)	166412-78-8	no	$> 5*10^{-4}$
Tributyl citrate (TBC)	77-94-1	yes	$5*10^{-5}$
Acetyl tributyl citrate (ATBC)	77-90-7	no	$> 5*10^{-4}$
Bisphenol A (BPA)	80-05-7	yes	$3*10^{-7}$
DEHTP (Bis-2-ethylhexyl terephtalate)	6422-86-2	no	$> 5*10^{-4}$
17β-estradiol (positive control)	50-28-2	yes	$1*10^{-11}$

By comparing the results from table 3, table 4 and table 5 some, but not all determined estrogen activities can be explained by detected substances in the migrates.

In the estrogen active migrates of samples T08c, T14a, T16, T17 and T18 no estrogen active substances could be identified by chemical analysis.

In migrates of the most estrogen active sample T02, the estrogen active UV-blocker benzophenone could be detected; however, the detected concentration only explains a small fraction of the detected estrogen activity. Based on the response curves for benzophenone and 17β estradiol in figure 2, benzophenone is approximately by a factor of 5×10^6 less estrogen active then the natural estrogen 17β estradiol. Therefore, the detected concentration of 0.3 µg benzophenone/cm² explains less than 1% of the determined estrogen activity of the sample. Therefore, additional unknown substances have contributed to the estrogen activity.

The same seems true for sample T9b. T9b migrates show an estrogenic activity of 67 pg EEQ/cm^2. However, T9b contains benzophenone only in low amounts of 0.38 µg/cm^2.Considering the discussed activity differences between 17β estradiol and benzophenone again less than 1% of the measured activity can be explained by the reported benzophenone concentration in the sample migrate.

In the migrates of the two estrogen active samples T05a and T05b, the well-known estrogen active substance BPA could be detected. In the ER-CALUX®, BPA is approximately by a factor of 30.000 less estrogen active than the natural estrogen 17ß-estradiol (see figure 2). Based on this factor, the estrogen activity of 54 pg EEQ/cm² detected in sample T05a can be well explained by a BPA concentration of 1.3 µg/cm², taking into consideration the uncertainty of a biological test method. Applying the same logic, the activity of 38 pg EEQ/cm² detected in sample T05b matches a BPA concentration of 1.1 µg /cm² detected by target analysis. In addition to BPA, TBC was detected in both samples but based on the response curve for TBC, shown in figure 2, the detected concentration would not significantly contribute to the total estrogen activity of the sample.

It was not always possible to link the observed endocrine activity with identified compounds. Either the chemicals in question cannot be detected by GC-MS because they may not be volatile or the substances are detected, but not covered by the NIST library. Moreover, among all possible endocrine active substances, analysis was performed for a limited set of substances by chemical analysis. Therefore, it is possible that the responsible substance cannot be detected. Thus, it would be very difficult to perform a quantitative risk assessment without an identification of the substance involved.

2.5. Risk Assessment for detected substances

The concentrations of the compounds following chemical trace analysis were analysed in the light of their potential effects on children.

For benzophenone, based on assumptions by RIVM [19], a maximum daily exposure of 1.4 µg/kg bodyweight can be calculated from the reported migration into the saliva solvent.

The calculation was done as described in the following:

i) sample T02: 0.33 µg/cm² in 60 min equivalent to 9.9 µg in 10 cm² in 180 min for a child of 8 kg bodyweight, a maximum daily uptake of 1.2 µg/kg.

ii) sample T09b: 0.38 µg/cm² in 60 min equivalent to 11.4 µg in 10 cm² in 180 min or for a child of 8 kg bodyweight, a maximum daily uptake of 1.4 µg/kg.

These estimated daily uptakes from toys, are still below the tolerable daily intake (TDI) of 30 µg/kg body weight per day, which was recommended by the European Food Safety Agency [36].

For DINCH, considering that the lowest reference value published for this compounds is 0.7 mg/kg bw/day [37], the exposure *via* toys is not expected to raise any safety concern for children.

Based on published data, ATBC does not seem to show estrogenic or androgenic activity but there are doubts concerning activation of the PXR-receptor pathway which could affect the metabolism of steroid hormones [2]. Therefore, no conclusion can currently be drawn on the endocrine-disrupting nature of ATBC, because no robust data are available on potential effects such as those on thyroid function.

Concerning TBC, there is also very limited toxicological data available to assess the endocrine effects for human health but based on similar chemical characteristics, an analogue approach seem plausible for systemic effects [38]. Therefore, as for ATBC, no conclusion can currently be drawn on the endocrine-disrupting nature of TBC.

For BPA, the modes of action for each of the adverse effects are not well described yet but estrogenic or anti-androgenic activities are compatible with the reported effects [34]. A maximum daily exposure of 4,9 µg/ kg bw/day can be calculated from the detected concentration of BPA in the saliva solvent in the toys T05.

The calculation was done as described in the following:

i) sample T05a: 1.3 µg/cm² in 60 min equivalent to 39 µg in 10 cm² in 180 min for a child of 8 kg bodyweight, a maximum daily uptake of 4.9 µg/kg.

ii) sample T05b: 1.1 µg/cm² in 60 min equivalent to 33 µg in 10 cm² in 180 min or for a child of 8 kg bodyweight, a maximum daily uptake of 4.1 µg/kg.

The daily uptake is just above the temporary tolerable daily intake (TDI) of 4 µg/kg body weight per day, which was recommended by the European Food Safety Agency (EFSA) in their re-evaluation of the risks of Bisphenol A taking into account endocrine effects [39].

3. CONCLUSION

By applying different *in vitro* bioassays, we were able to detect significant estrogenic activity in at least one part of eight out of 18 tested toy items.

The tested samples were intended to reflect a realistic product status after delivery to the consumer. Therefore, no direct conclusions on the origin of estrogen active substances can be drawn based on these results. Some endocrine active substances might have been introduced by contaminations from packaging or during transport, as samples have been ordered online and were mostly delivered in recycled cardboard boxes, which are known to frequently contain endocrine active substances, such as BPA. To ensure that detected substances originate from the product, samples would have to be drawn directly at the production site and transported under controlled conditions, e.g. wrapped in aluminium foil.

In two of the tested samples, the well-known endocrine-active substance BPA could be identified as the main source of the detected estrogen activity. Based on the results, the exposure to BPA by mouthing of these toys could be above the temporary TDI, that EFSA has calculated for bisphenol A, demonstrating that some toys could significantly contribute to the total exposure to BPA of babies and infants.

For seven other estrogen active toy items (including the most estrogen active sample in the screening), the source of the estrogen activity could not be explained by chemical analysis for known or suspected endocrine active substances in plastics, indicating that the effect was caused by currently unknown endocrine active substances.

However, compared to the exposure to natural estrogens in food (e.g. phytoestrogens), the total uptake of estrogen activity by mouthing of toys seems to be low based on the results in this study.

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Figure1

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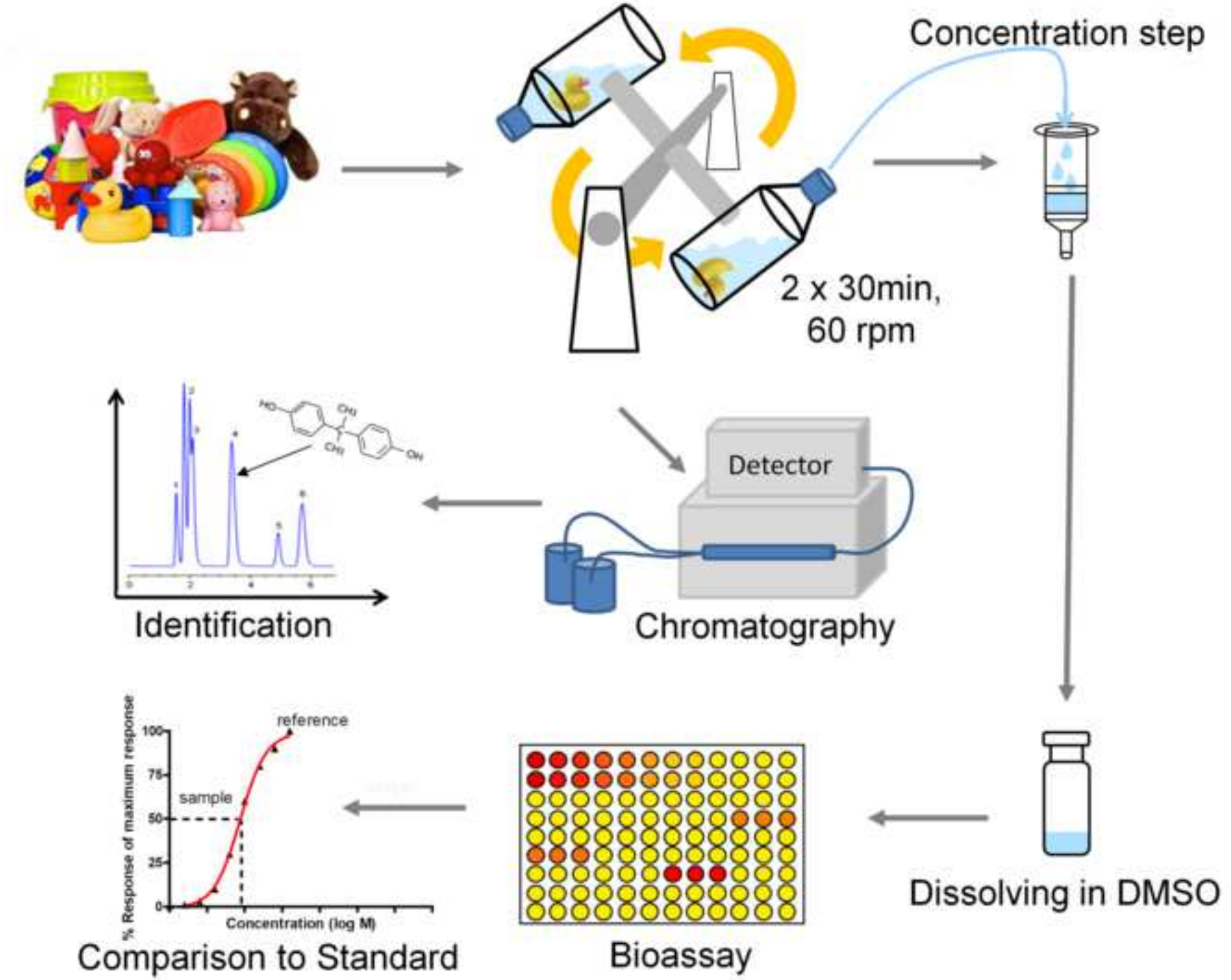

Figure2

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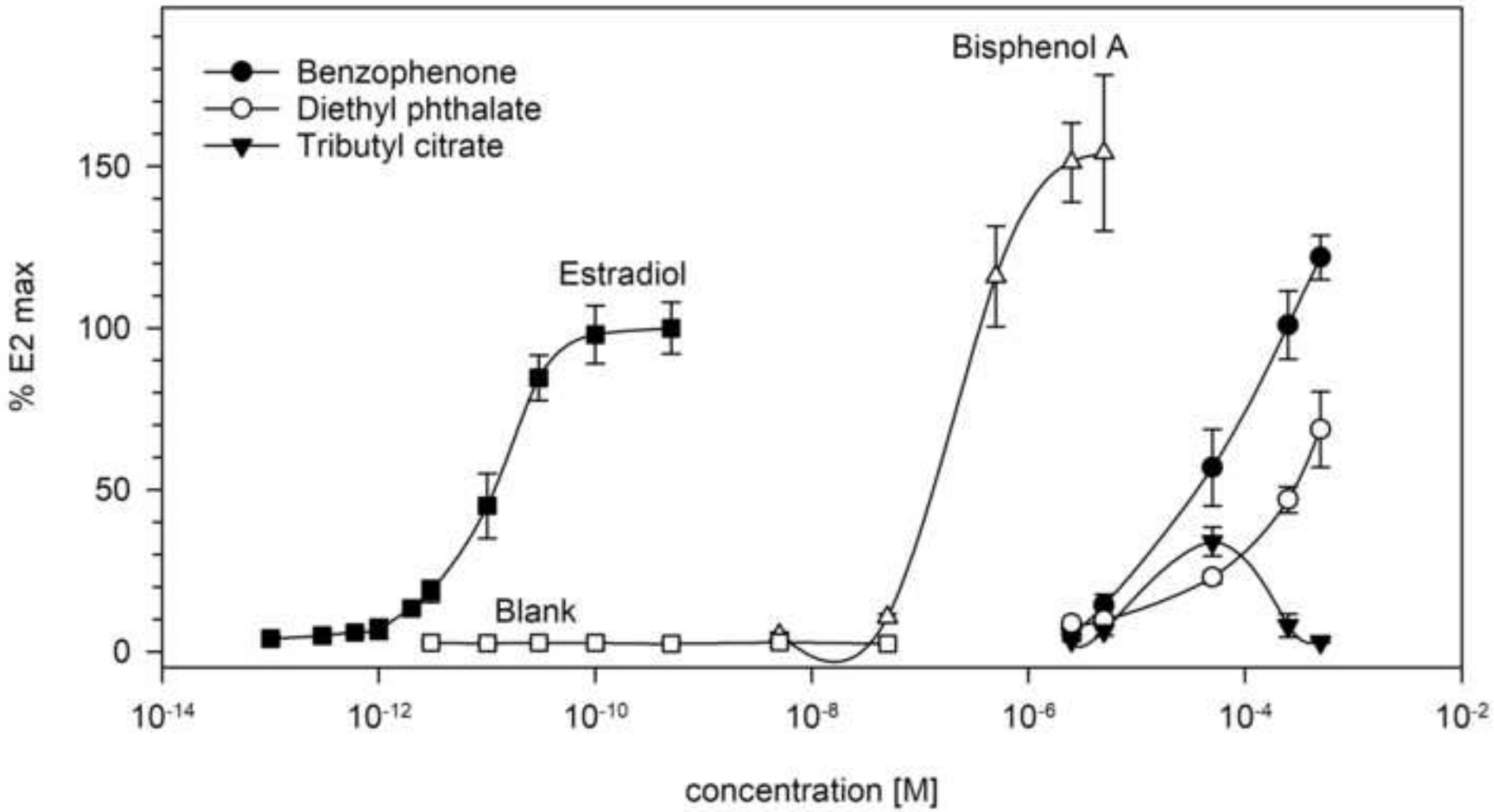

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